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14. ABSTRACT
The purpose of this proposal is to detect the role of Stat3 activation during prostate cancer progression. A multifaceted approach is being used to accomplish the proposed research goals. Significant progress was made in support of Task 1 in that we were able to overexpress activated Stat3 (Stat3C) in tow human prostate cell lines. Both lines were characterized and at least on line (MDAPLa 2b) exhibits molecular alterations consistent with Stat3 activation. In support of Task 2, we have begun characterization of the Pb.Stat3C transgenic line and the preliminary histopathological findings indicate that expression of activated Stat 3 may confer a neoplastic phenotype. The proposed bigenic cross (Pb.Stat3C x BK5.IGF-1) has also been initiated as well as an alternate strategy due to the limited fertility of the BK5.IGF-1 transgenic line and the apparent compromise in viability of the bigenic offspring. Our results to date have been promising and should further our understanding of the role of Stat3 activation in prostate cancer progression.

15. SUBJECT TERMS
STAT3, EMT, BK5, IGF-1, PB, PIN, MMP, MT1-MMP, PTEN, prostate, e-cadherin

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Introduction

Signal transducer and activator of transcription 3 (STAT3) has been implicated in many processes including development, differentiation, immune function, proliferation, survival and epithelial to mesenchymal transition (EMT) (1-4). In addition, constitutive activation of STAT3 has been reported in many cancers, including prostate cancer. Different studies in prostate tissues indicated that STAT3 activity was high in prostate carcinomas as compared to normal prostate tissues. Some of the studies suggest that STAT3 activation happens early and persists through prostate cancer progression and others suggest that STAT3 is activated only in prostate cancer tissues and the activation levels of STAT3 positively correlate with malignancy of tumors and increased Gleason scores (3, 5, 6). Although these studies have led to different conclusions about STAT3 activation in early prostate cancer, they all agree that STAT3 activation is elevated in malignant tumors as compared to normal prostate tissues. In addition, analysis of the three well-characterized prostate cancer lines DU145, PC3, and LnCAP indicated that STAT3 is activated in all three of these cell lines. DU145 had the highest levels and LnCAP (the least malignant of the three) had the lowest levels of STAT3 activation. Preliminary data from our laboratory suggests that STAT3-C expression increases invasiveness in a mouse keratinocyte system. During EMT many proteases are upregulated and cell adhesion molecule expression is altered to allow for more invasive phenotypes (7-10). In this proposal, we will study the role of STAT3 activation during prostate cancer progression. Collectively, the above pieces of evidence combined with the fact that STAT3 activation appears to be involved in EMT and has been linked to increased MMP-7 and MT1-MMP expression in prostate cancer cells gave rise to our hypothesis that:

STAT-3 activation regulates the expression of genes involved in EMT, including matrix metalloproteinases (MMPs), leading to increased invasive and metastatic potential in prostate cancer.

The expected results are that overexpression of STAT3-C will result in EMT, leading to increased MMP expression, and finally, resulting in increased invasiveness and metastasis *in vivo* and *in vitro*.

Body, Key Research Accomplishments, Reportable Outcomes, and Conclusion:

The following progress was made in support of Task1a and b.

Task 1: Determine whether over-expression of activated STAT3 (STAT3c) in LnCap cells stimulates EMT.

LnCap cells were stably transfected using a CMV-STAT3c vector, [Murine Stat3 was cloned into RcCMV-Neo (InVitrogen) tagged at the 3′ end with a FLAG epitope. The Stat3-C construct was made by site-directed mutagenesis (Quick-Change by Promega) using primer pairs 5′-GCTATAAGATCATGGATACCCATCCTGGTGTCTCC]. We were able to obtain five clones, which were then characterized. Western blot analysis was performed on cell lysates prepared from each clone as well as the parental LNCaP line to evaluate the expression of total STAT3 protein and to confirm the presence of the Flag tag. As shown in Figure 1, although a few of the clones appeared to have an increase in expression of total Stat3 protein only one of the clones was positive for expression of the flag tag indicating successful transfection of the entire STAT3c vector. Further characterization was performed on the positive clone, hereafter referred to as LNCaP- STAT3C.

Western blot analysis was performed on cell lysates prepared from cell cultures at confluency to examine the expression of several proteins that have been shown to be critical to cell proliferation, migration and anchorage. The results of these experiments are shown in Figure 2. There were no significant alterations in the expression of Cyclin D1, VEGF, integrin α6, or MMP2 and MMP9. In addition we were unable to detect expression of MMP7 in either the LNCaP parental line or LNCaP- STAT3C (data not shown). MMP caseinase and gelatinase activity were assessed by gel zymography. The results are shown in Figure 3. We were unable to detect caseinase activity in either cell line (Fig. 3A) and there was no difference in gelatinase activity (MMP2, MMP9) between the two lines.

The following progress was made in support of Task 2a and b.

Task 2: Determine whether STAT3 activation increases invasiveness.

The invasive properties of the two cell lines were compared using an *in vitro* migration assay. The results are shown in Figure 4. There was no difference in migration through matrigel between the two lines. Review of the preliminary results indicated that overexpression of STAT3c did not appear to confer an invasive phenotype on the parental LNCaP cell. Thus, rather than progressing to part b of Task 2 which is the assessment of the invasive properties of the LNCaP-STAT3c clone in vivo, we elected to pursue an alternate strategy.

The *in vitro* experiments were repeated using a different prostate cancer cell line that was developed at MD Anderson Cancer Center, MDA PCa 2b (11). The vector described above was used to transfect the cells and three clones were obtained. Initial characterization of the clones by Western blot analysis revealed that all three clones were positive for the Flag-tag and had a slight increase in total STAT3 protein. Phosphorylated STAT3 was slightly detected in the original MDA PCa line and in the transfected clones. Analysis of MMPs 2, 7, and 9 revealed an increase in protein expression of MMP2 but no difference in the level of MMP9. We were unable to detect expression of MMP7 in any of the lines (data not shown). In addition, there was an apparent decrease in the level of E-cadherin in 2 of the 3 clones (MDA Pca-STAT3c1 and 2) that correlated with increased levels of MMP2. We are in the process of completing the characterization of the phenotype of the MDA PCa-STAT3c clones. If, as expected, transfection of the MDA PCa cell line confers an invasive phenotype then this line will be used to examine whether this phenotype is maintained in vivo as described in Task 2a.b.

The objective of Task 2b is to determine whether expression of probasin-STAT3c in the background of BK5.IGF-1 transgenic mice leads to higher expression levels of MMPs in prostate tissues and development of more invasive tumors. For the first part of this task (2b.a.) we had proposed to develop double transgenic mice that were hemizygous for both the BK5.IGF-1 transgene (+/-) and the Pb.Stat3c transgene (+/-). We began with six sets of breeders, each consisting of a BK5.IGF-1 transgenic male paired to a Pb.Stat3C female. To date, only one female has successfully given birth and each litter has been unexpectedly small. Although the breeding colony has been expanded it may be difficult to produce a sufficient number of the expected genotypes to accomplish the research goals since the male double transgenic animals represent ~ 10% of the total number of animals born.

Hence we have employed an alternate strategy. We have available in our facility PTEN knockout mice on an ICR background. It is commonly known that loss of one PTEN allele occurs frequently in prostate cancer, taking place in as many as 70-80% of primary tumors (12-15) and homozygous inactivation of PTEN is associated with advanced disease and metastasis (16, 17). The tumor suppressor PTEN is a negative regulator of signaling through the IGF-1 receptor by inhibiting the capacity of PDK1 to activate AKT (18-20). In addition it has been shown, at least in tissues other than the prostate, that there are a downstream link between mTOR and Stat3, in which mTOR is required for maximal activation of STAT3. Thus, we expect that the loss of PTEN may enhance the onset of tumor development compared with single transgenic and it is hoped that the additional perturbation of activated STAT via the Pb.STAT3c transgene will enhance the development and progression of prostate tumors. Breeding has begun to produce male mice hemizygous for the Pb.STAT3c transgene and the PTEN knockout allele. To date there appears to be no compromise in reproduction.

As part of Task 2b.c. we have begun the initial characterization of the prostate of aged hemizygous Pb.STAT3C male mice. Necropsy and prostate dissection was performed on 10 mice that ranged in age from 6 to 7 months. The individual lobes were harvested and snapfrozen for biochemical analysis or the genitourinary tract was removed intact and fixed in phosphate-buffered formalin. Representive sections of the preliminary findings are shown in Figure 6. The morphology of the anterior prostate (AP) was essentially normal and consistent with the morphology observed in nontransgenic males of the same age. The glands of the ventral prostate (VP) were predominantly normal in appearance but epithelial hyperplasia was occasionally observed. Although the initial sample size for histology study was small (n=4), examination of the dorsalateral prostate (DP) revealed that at least one animal presented with a mix of atypical epithelial hyperplasia and cellular changes consistent with prostatic intraepithelial neoplasia (PIN). Further analyses will have to be performed to confirm the prevalence and etiology of the cellular alterations in the DP.

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Legends and Figures:

Figure 1. Western blot analysis of the parental LnCaP cell line and LnCaP -Stat3C transfected clones (from LnCaP Stat3C 1 to 5). Protein lysates were prepared from cultured cells and analysed with antibodies against (A) the Flag-tag and (B) total STAT3.

Figure 2. Analysis of protein expression by western blot using antibodies against the Flag-tag, MMP2, MMP9, Cyclin D1, Integrin α-6, and VEGF (vascular endothelial growth factor) in the LnCaP cell line and the LnCaP -Stat3C clone.

Figure 3. Zymography gel assay for casinase (3-A) and gelatinase (3-B) activity in both the parental LnCaP cell line and the LnCaP -Stat3C clone. Lysates were prepared from cultured cells, separated by SDS-PAGE gels containing either casein or gelatin and analysed for activity after removal of SDS.

Figure 4. In vitro migration assay through Matrigel membrane matrix. Assay was performed under two different Matrigel concentration (10ug and 50 ug) for both lines, LnCaP and LnCaP -Stat3C clone.

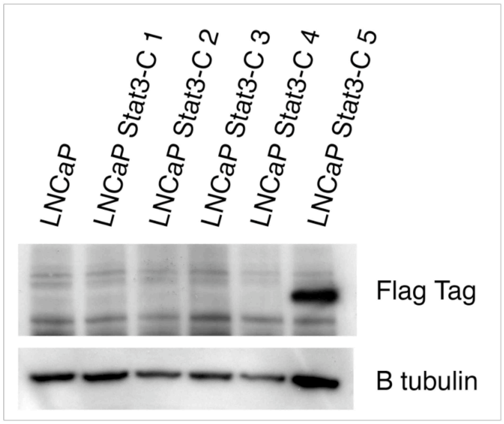
Figure 5. Western blot analysis of MDA PCa 2b cells and Stat3C-transfected clones (MDA PCa 2b-Stat3C 1 to 3). Protein lysates were prepared from cultured cells and analysed with antibodies against the Flag-tag, total Stat3, phospho-Stat3, MMP2, MMP9, and Cyclin D1, e-cadherin)

Figure 6. Histopathology of hemizygous Pb.STAT3c transgenic male mice ranging in age from 6 to 7 months. (A) Section of anterior prostate (AP) and seminal vesicle (SV). Note the defined single stratum of luminal epithelial cells with normal recurrent mucosal folds projecting into the gland characteristic of the normal AP. No structural or cellular

atypia is evident. (B) View of normal entral prostate lobe (VP) with characteristic flat luminal edges and focal tufting. (C) Section of VP lobe with areas of hyperplasia; note the increase in epithelial tufting, but the otherwise normal appearance of the cells. (D) Section fo normal Dorsalateral prostate (DP) lobe; note normal glands lined by simple columnar epithelium with moderate degree of infolding. (E) Section of DP with prostate intraepithelial neoplasia (PIN). Luminal epithelial cells show a cribriform growth and the formation of many intra luminal small glands is evident. (F) Magnification of section designated by arrow in (E) showing cellular atypia, the presence of kariomegalia, kariocytomegalia, nuclear atypia, and cells with the presence of one or more prominent nucleoli. All sections were stained by hemotoxylin and eosin. Magnification of A,B is 4X, magnification of C-E is 10X, magnification of F is 20X.

Figure 1

1-A



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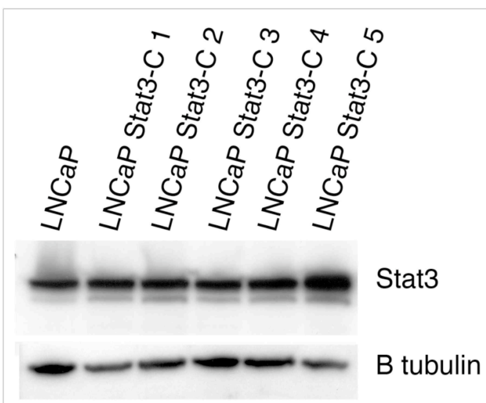


Figure 2

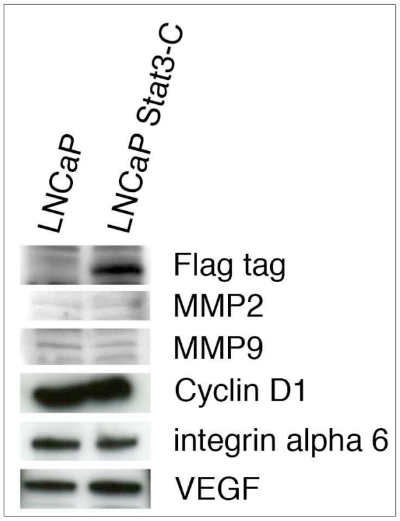
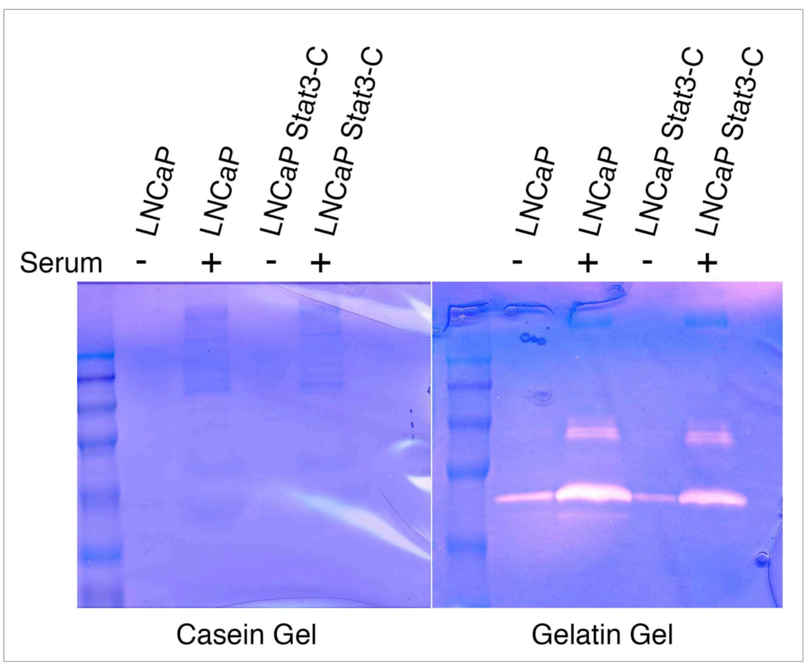


Figure 3



3-A 3-B

Figure 4

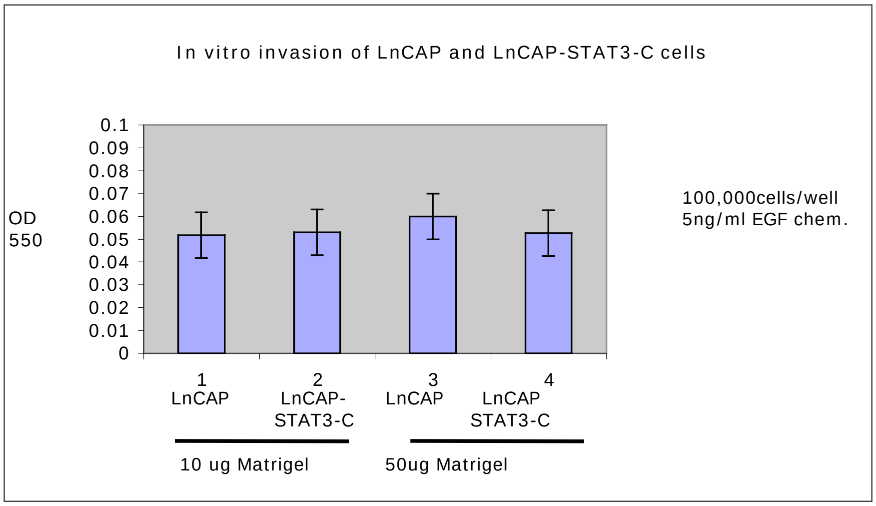


Figure 5

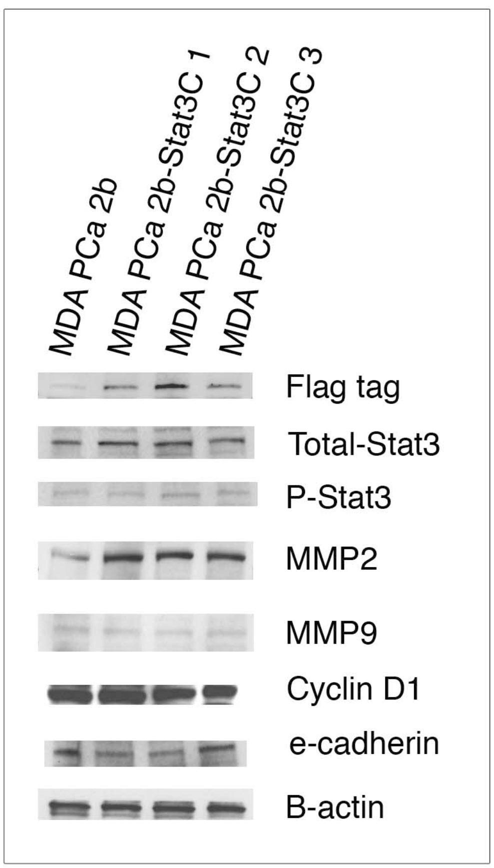


Figure 6